

Preparation of Quantum Dot Bioconjugates as Fluorescence Probe for Targeting and Visualization of Cells

Hücrelerin Hedeflenmesi ve Görüntülenmesi için Floresans Prob Olarak Kuantum Nokta Biyokonjugatlarının Hazırlanması

Research Article

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ABSTRACT

Here, the bioconjugation and application of water-soluble quantum dots (QDs) for targeted cellular imaging were investigated. Human anti-c-ErbB2 antibodies were bound to QDs for the targeting of these nanostructures towards the cancer cells. QDs and anti-c-ErbB2/QD bioconjugates were characterized by fluorescence and UV-Vis spectroscopy and hydrodynamic sizing. The effect of QDs and anti-c-ErbB2/QD bioconjugates on cell viability was evaluated using an MTT assay. To observe targeting efficiency of anti-c-ErbB2/QD bioconjugates, fluorescence microscopy and was performed. Higher cell affinities were obtained by anti-c-ErbB2-conjugated QDs which is an evidence that these are well-suited for targeting and imaging of cells.

Key Words

Quantum dots, bioconjugation, anti-c-ErbB2, cell targeting.

ÖZ

Burada, hedeflenmiş hücresel görüntüleme için suda çözünür kuantum noktaların (QD) biyokonjugasyonu ve uygulamaları gerçekleştirilmiştir. Kanser hücrelerine nanoyapıların hedeflenmesi için anti-c-ErbB2 antikoru QD'lara bağlanmıştır. QD'lar ve anti-c-ErbB2/QD biyokonjugatları floresans/UV-Görünür bölge spektroskopisi ve hidrodinamik boyut ölçümleri ile karakterize edilmiştir. Hücre canlılığına QD'lar ve anti-c-ErbB2/QD biyokonjugatlarının etkisi MTT denemeleri kullanılarak gerçekleştirilmiştir. Anti-c-ErbB2/QD biyokonjugatlarının hedefleme etkinliğini gözlemek için, floresans mikroskopi kullanılmıştır. Hücreleri görüntüleme ve iyi gerçekleştirilmiş hedeflemenin ispatı olarak anti-c-ErbB2 konjuge edilen QD'lar ile daha yüksek hücre afiniteleri elde edilmiştir.

Anahtar Kelimeler

Kuantum noktalar, biyokonjugasyon, anti-c-ErbB2, hücre hedefleme.

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INTRODUCTION

Development and design of new fluorescence probes for targeting and imaging have great attention in biomedical technologies and biomedicine. Scientists focused on developments of targeting and bioimaging probes in improved properties [1-3]. Fluorophores, which are used in visualization of cells, can be divided some main groups such as organic dyes, conjugated polymers and inorganic-organic nanostructures [2-7].

Semiconducting nanoparticles (quantum dots: QDs) are [8,9] new type of markers which are applied to screen biostructures using optical sensing technologies. QDs have characteristic properties in comparison to fluorescence dyes, such as possibility to synthesize in different excitation/emission wavelengths, long fluorescence life time, resistance to metabolic degradation and high quantum yields [10-12]. Furthermore, QDs are colloids with similar size to large proteins, dispersed in an aqueous solvent and covered with organic structures to stabilize their dimensions [13]. Also the surfaces of QDs can be modified various functional groups such as amine and carboxyl [2,3]. The solubility of QDs in aqueous media are important for their applications on biological samples. Also the solubility of QDs affect bioconjugation conditions with ligands. Several cell-targeting ligands have been reported to modify QDs in previous studies. Bharali et al. synthesized hydrophilic InP Quantum Dots and modified with folic acid. The delivery of bioconjugates into folate-receptor-positive cell lines was demonstrated and receptor-mediated delivery was proved [14]. In another study, dendrimer-modified quantum dots were synthesized and were conjugated with Arginine-Glycine-Aspartic Acid (RGD) by Li et al. [15]. The designed bioconjugates injected into nude mice loaded with melanoma (A375) tumor xenografts via tail vessels. Furthermore, HER2 monoclonal antibody conjugated RNase A-associated CdTe quantum dot cluster (HER2-RQDs) nanoprobe was formed and applied for targeted imaging and therapy of gastric cancer cells (MGC803) [16]. In our previous studies, QDs were covered with amine-terminated polyamidoamine (PAMAM) dendrimer and HER2 receptor-mediated targeting efficiency of antibody labelled PAMAM/QD conjugates was proved [2]. In another study, synthesis, characterization, bioconjugation with anti-HER2, and application of

water-soluble thioglycolic acid TGA-capped CdTe/CdS quantum dots (TGA-QDs) were carried out [3].

Human epidermal growth factor (EGF) receptor 2 (ErbB2) has been well known important cell surface protein for breast cancer. c-erbB2 is overexpressed in cancer cells especially breast cancer. Anti-c-erbB2 was used to detect the well-known breast cancer biomarker c-erbB2 in previous studies [17,18]. In this study, anti-c-ErbB2 antibody and carboxyl-QDs were used to form anti-c-ErbB2/QD bioconjugates for targeting of cancer cells. First, the optimization of loading amount of Ab to QDs were carried out. Then, the effect of bioconjugates on cell viability was tested. Finally, fluorescence imaging of cells using anti-c-ErbB2/QDs conjugates was investigated.

MATERIALS and METHODS

Materials

Rabbit monoclonal antibody to human c-ErbB2 protein was purchased from Diagnostic Biosystems. (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2-(N-morpholino)ethanesulfonic acid (MES), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS) were purchased from Sigma. Phosphate buffer solution was prepared at the following composition: 8.0 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na₂HPO₄ and 0.24 g/L KH₂PO₄. All other chemicals were analytical grade.

Instrumentation

Eppendorf thermomixer comfort (2.0 mL) was used to keep temperature at 25°C during conjugation of antibody with QDs. Thermo electron corporation HERA cell 240 CO₂ incubator was used for cultivation of cancer cells. Emission and absorbance spectrum of QDs were recorded with NanoDrop 3300 Fluorospectrometer and NanoDrop ND 1000 spectrophotometer, respectively. Bi-orad Model 680 microplate reader was used to read absorbance in MTT assay. Average particle size and size distribution of the QDs/anti-c-ErbB2 bioconjugates were measured using Dynamic Light Scattering (DLS, Malvern Instrument, U.K). The QDs and anti-c-ErbB2/D bioconjugates were diluted with PBS before measurements.

Bioconjugation Reactions of QDs and Anti-c-ErbB2

The synthesis of thioglycolic acid modified carboxyl-QDs and bioconjugates of anti-c-ErbB2 with QDs were carried out according the literature [1-3]. Briefly, 0.2 mL of QD solutions were mixed with 0.025 mL of EDC (0.2 M in MES buffer, 25 mM pH, 6.0), 0.025 mL of NHS (0.05 M in MES buffer, 25 mM pH:6.0) and carboxyl groups of QDs were activated for 15 min at room temperature. Then anti-c-ErbB2 antibody (25 µg/mL) was added and reaction was completed in 2 h at room temperature. Finally, the bioconjugates were purified using PALL corporation, VWT nanosep 300K omega membrane filter (MWCO: 300 KD) and washed with PBS for 4 times.

Cell Culture

MCF-7 (breast cancer), NIH-3T3 (mouse fibroblast), Caco-2 (colon cancer), A-549 (human lung cancer) and HEP-G2 (liver cancer) cell lines were provided from German Collection of Microorganisms and Cell Cultures (DSMZ). MCF-7 cells were cultivated in Minimum Eagle's Medium (MEM) media containing 10% Fetal Calf Serum (FCS), 1.0% Na-pyruvate, 1.0% L-glutamine, 10 µg/mL insulin, 1.0% non-essential amino acids and 1.0% penicillin/streptomycin (P/S). NIH-3T3 cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% Newborn Calf Serum (NCS) and 1.0% P/S. Caco-2 cells were cultivated in MEM containing 20% FCS and 1.0% P/S. A-549 and HEP-G2 cells were incubated in DMEM containing 10% FCS and 1.0% P/S. All cells were cultivated in medium at 37°C in humidified environment of 5.0% CO₂.

In vitro Cytotoxicity

(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction test were used to determine cytotoxicity of QDs and anti-c-ErbB2/QD bioconjugates. Cells were seeded out in 96-well-tissue plates. The cultivation took three days at 37°C, 5.0% CO₂ and 100% air humidity. After this cultivation time the wells were confluent populated. The medium was removed and the cells were incubated with QDs samples solved in medium. Then the samples were carefully removed. This removal has to be very exhaustive. Otherwise reducing medium or

sample ingredients are able to react with MTT solution and cause false-positive results. 110 µL freshly prepared MTT solution (10%) in cell corresponding medium were added per well. The cells were incubated at 37°C, 5.0% CO₂, 100% air humidity for 4 h. While this incubation time the formazan complex was produced inside the cells. To release the purple colored salt 100 µL SDS solution in 1.0 M HCl (10%) were added per well and the plates are incubated at 37°C, 5.0% CO₂, 100% air humidity for 24 h. Finally, the plates were read out in a plate reader (spectrophotometer). A decrease of living cell amount causes a decrease of metabolic activity. This activity is correlated directly with the amount of produced purple formazan (at 570 nm).

Cell Imaging

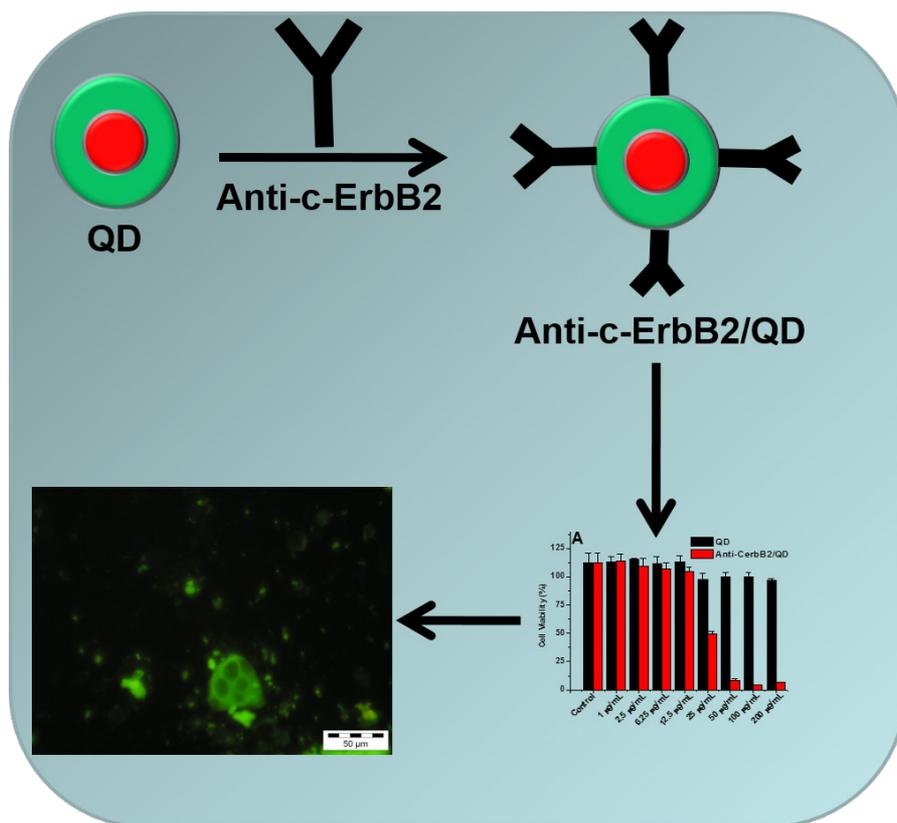
Each cells (MCF-7: 8000 cfu/mL, NIH-3T3: 10000 cfu/mL, Caco-2: 8000 cfu/mL, A-549: 8000 cfu/mL and HEP-G2: 10000 cfu/mL) were cultivated in 96-well plate with 5.0% CO₂ in their respective medium at 37°C as described before. After 72 h cultivation, adherent cells were washed twice with PBS. And 0.1 mL of QDs, diluted with PBS, was added into the wells. The cells were allowed to be incubated with the QDs for 4 h and were washed 2 times with PBS after incubation. The cell imaging studies were carried out using Olympus IX50 fluorescence microscope with 10x and 40x magnifications. The preparation and applications of anti-c-ErbB2/QD bioconjugates were depicted in Scheme 1.

RESULTS and DISCUSSION

Characterization of Anti-cErbB2/QDs Bioconjugates

UV-VIS Spectrometer and fluorescence spectrophotometer were used for characterization of optical properties of QDs. Figure 1 shows the UV-VIS absorption spectrum of QDs in aqueous solution together with the emission spectra after excitation at 470 nm. QDs exhibit visible emission at 550 nm.

The fluorescence intensity of emission was found to decrease with the increase in antibody amount. These results are consistent with the previous studies. Figure 2 shows the



Scheme 1. Schematic representation of modification and application of QD bioconjugates.

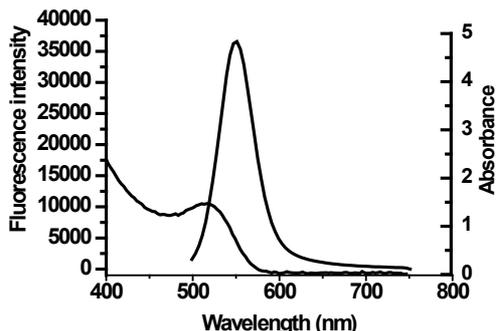


Figure 1. Absorption (A) and emission (B) spectra after excitation at 470 nm of QDs dispersed in PBS.

emission spectra of QDs after loading of varying concentration of antibody. Addition of targeting ligands to QDs results in the change of biological or chemical and physical properties of QDs. When stoichiometric ratio of QDs to capping agent such as folic acid or antibody decreases the PL intensity decreases [19,20]. Manzoor et. al explained two ways to occur this decrease. First, the light to reaching to QDs was filtered out by the surface conjugated capping agent due to the absorption of excited light. Secondly, the non-radiative energy transfer and/or hot electron exchange can be carried out between QDs and capping agent [19]. After consideration of these results, 28.5 µg anti-c-ErbB2 antibody/mg QD

was used for furthermore experiments to achieve both cancer targeting and high signals.

To investigate diameter effect of antibody amount, three different concentrations of anti-c-ErbB2 was loaded to modify QDs. Figure 3 depicts the DLS results of bioconjugates. The hydrodynamic diameters of QDs, 11, 28.5 and 62 mg anti-c-ErbB2/mg QD loaded conjugates were 10.90 ± 1.13 , 30.44 ± 3.15 , 50.75 and 58.77 nm (n: 3), respectively.

In the MTT assay, cells were grown in corresponding medium for 72 h. Then it was

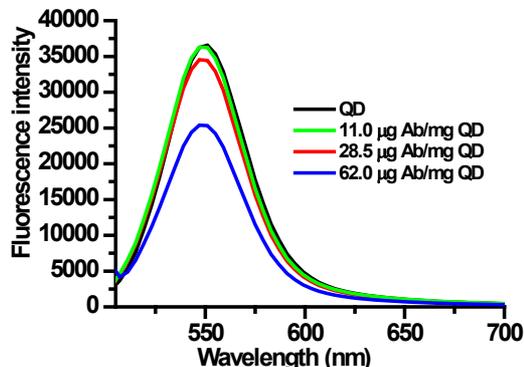


Figure 2. Effect of antibody amount on the fluorescence intensity of QDs.

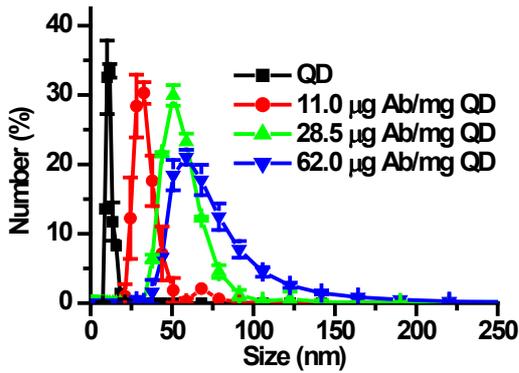


Figure 3. DLS of QD and anti-c-ErbB2/QD bioconjugates.

removed and medium containing QDs and bioconjugates at a concentration ranging from 0 to 200 µg/mL was added and cells were incubated in CO₂ incubator for 4 h. As shown in Figure 4, the cell viability was decreased with increasing QD concentrations. NIH-3T3 was not resistant to 200 µg/mL of QDs unlike HEP-G2, A549, MCF-7 and Caco-2 cells. After addition of the biomolecule to structure, the concentration of QD affected all cell lines. Cells kept their viability up to the 12.5 µg/mL of QD. The results were not surprising, because decrease of anionic groups on the surface of QDs

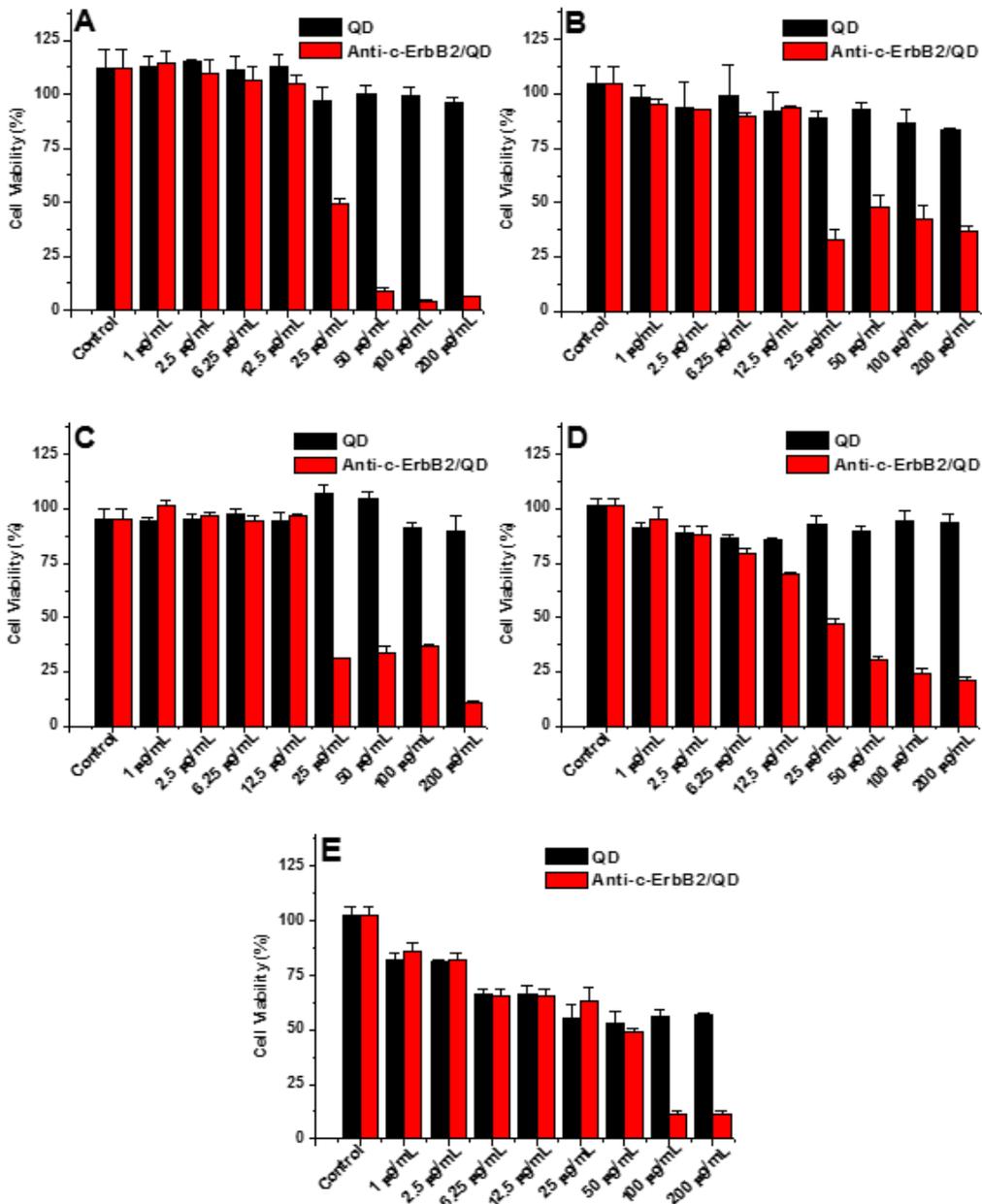


Figure 4. In vitro cell viability of cancer cells treated with varying concentrations of QDs and anti-c-ErbB2/QD conjugates for 4 h (A: HEP-G2; B: MCF-7; C: A549; D: Caco-2 ; E: NIH-3T3).

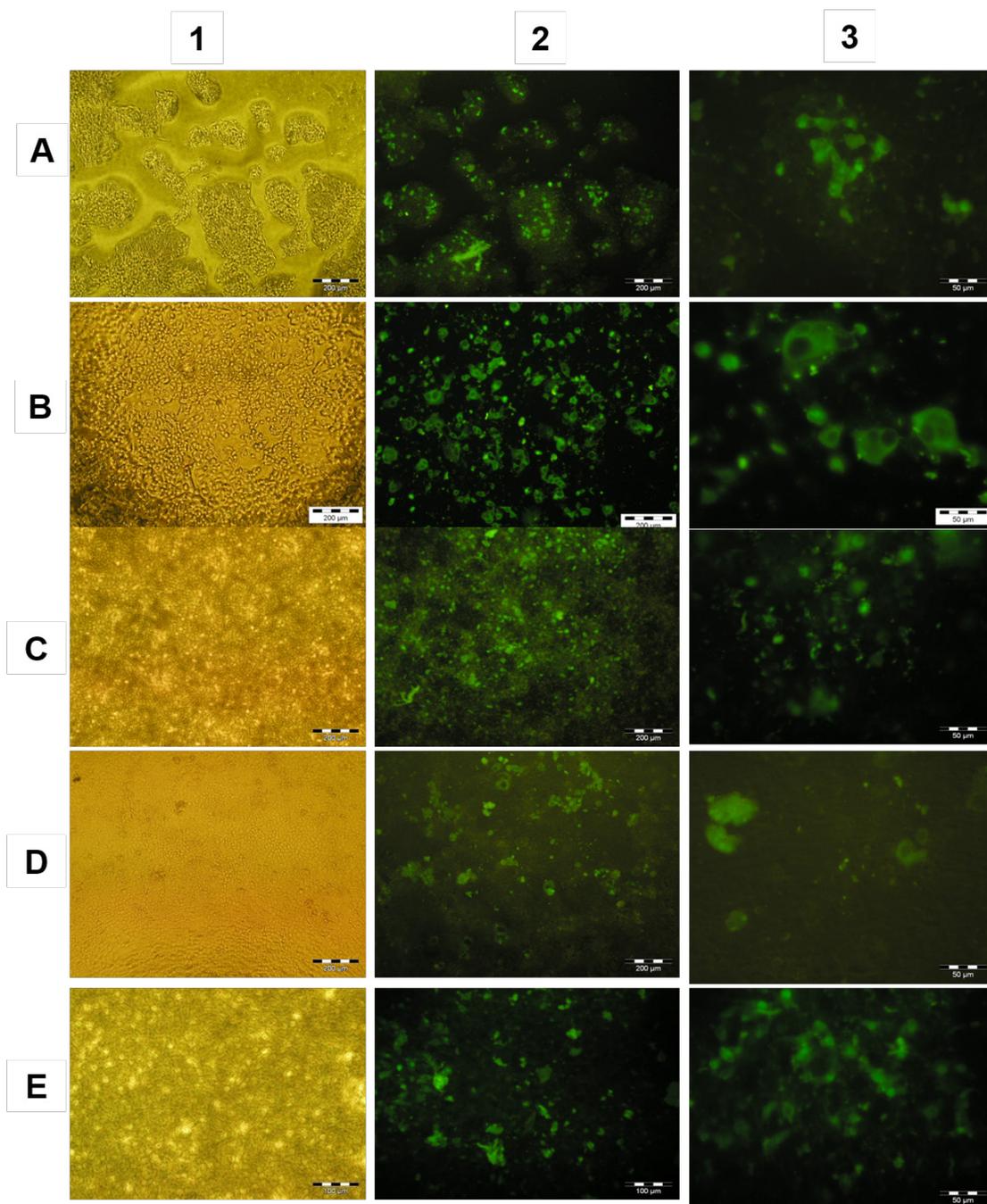


Figure 5. Bright field images of cells with 10x magnification (1) and fluorescence microscopy images of cells with 10x (2) and 40x (3) magnification (cells were treated with QD-anti-c-ErbB2 bioconjugates for 4 h; (A): HEP-G2; (B): MCF-7; (C): A549; (D): Caco-2; E: NIH-3T3).

increases the cytotoxicity [20].

Fluorescence microscopy of anti-c-ErbB2/QD was performed after incubating bioconjugates with A549, MCF-7, HEP-G2, Caco-2 and NIH-3T3 cells for 4 h at 37 °C. After modification of the QD surface with antibodies, cellular uptake through endocytosis was carried out [3]. Microscopy images of cells were presented in Figure 5.

Anti-c-ErbB2/QD nonspecifically bound to healthy cells (NIH-3T3 as a model in this assay), but the fluorescence signals and the visualization of cells were much lower than those in A549 cells.

CONCLUSIONS

The QDs and anti-c-ErbB2/QD bioconjugates were synthesized and applied to image cancer cells. For

this aim, anti-c-ErbB2 was bound to QDs, including carboxyl groups on their surface, via EDC/NHS coupling reaction. The formed anti-c-ErbB2/QD bioconjugates were characterized optically and applied to image cancer cells. The effect of anti-c-ErbB2/QD bioconjugates on cell viability was tested by MTT assay. Taken together, these results suggest that anti-c-ErbB2/QD can be used as fluorescent probes for cellular imaging of cancer cells and in vivo imaging applications, such as biomarker detection.

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